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CHEMICAL CORPS BIOLOGICAL LABORATORIES 465
Fort Detrick, Maryland

Localization of an ECHO-9 virus in tissues of infected suckling mice by means of fluorescent antibodies.

by R. Gaedeke.

Earlier investigations have shown that ECHO-9 virus strain K 696 (5-7) isolated on monkey kidney cell cultures has no apparent pathogenic effect on the suckling mouse. Such animals did, however, yield a virus titer with a $TCID_{50}$ at dilutions of 10^5 to 10^6 from skeletal muscle tissue following inoculation of the virus strain passed through tissue culture; in addition, a drop in muscular potassium and a shift in the protein/ nucleic acid extraction quotient in favor of a relatively better protein extractability were noted (3). These findings may be interpreted as an inapparent infection with textural disorders in skeletal muscle tissue without evident impairment of function or structure. Successive passage of the virus through the suckling mouse produces not only morphological correlates of myositic and myodenerative irritations, but also meningitic reactions and, ultimately, encephalitic lesions together with paralytic manifestations in infected animals.

Subsequent studies recounted in this paper proceeded from two problems: 1. Demonstration of ECHO-9 virus in all stages of ascending pathogenicity for the suckling mouse from the inapparent infection to the paralytic disorder was to be tested.

2. In addition, the extent to which the pathogen could be localized in the musculature and central nervous system as well as the tissual structures favored by it were to be established.

We had found previously, by labeling the ECHO-9 virus with fluorescent antibodies and by electron-microscopic studies in collaboration with Dostal, Gruenewald, Mittelstrass and Sauthoff (2), that the virus is demonstrable in the cytoplasm of infected cells of monkey kidney cultures, but not in the region of their nuclei.

Our investigations, especially the production of fluorescein-isocyanate and its linkage to antibodies, were based on the method of Coons and Kaplan (1). We used an antiserum from a rabbit injected intramuscularly with $3 \times 10^{7.8}$ $TCID_{50}$ of ECHO-9 virus suspension from

non-cellular supernatants of rhesus monkey kidney cell cultures 6 times at intervals of 6 to 8 days. The neutralizing antibody titer of the resultant immune serum was 1:1024 against 100 $TCID_{50}$. The examined tissue material came from suckling mice which had been inoculated sub-cutaneously with $3 \times 10^{4.5}$ $TCID_{50}$ of the virus on the 1st or 2nd day of life. We tested several animals in every instance; they had been infected either with the K-696 strain passed through tissue culture or with the 1st to 6th suckling mouse passage of this pathogen. The animals were sacrificed 3 or 6 days after infection; the entire brain as well as skeletal muscle tissue from the iliopsoas region and from proximal parts of the lower extremities were prepared as deep-frozen sections of 12-15 microns. Air-drying of the preparation was followed by attachment of fluorescent antibodies according to Coons and Kaplan's instructions.

Results

After infection with the apparently non-pathogenic original virus strain, the morphologically inconspicuous muscle fibers of the skeletal muscle tissue (Fig. 1, a-h) reveal a very discrete fluorescence in the form of isolated longitudinal lines resembling strings of pearls; identical observations were made after the first and second suckling mouse passage of the pathogen. Along with the increase in the pathogenic effect of the virus on its host, accompanied by deviation from the normal morphology -- especially after the development of Zenker's muscle de-generation --, green fluorescence intensified considerably during subsequent passages. Even then, the fluorescent regions retained their longitudinal orientation within the muscle fibers, corresponding to the sarcoplasmic structures.

The central nervous tissue (Fig. 2 a-f) revealed fluorescence starting with the second suckling mouse passage of the virus. It was limited initially to isolated meningeal regions. The fine texture of these brains had shown increased infiltration of the soft meninges by large elements with round nuclei. During subsequent passages, an accumulation of fluorescent cells was noted there, spreading ultimately to the cerebral parenchyma, analogously to the increasing cellular-infiltrative reactions in the meninges. Here we noted a linkage of fluorescent particles (and, with them, of the antigen coupled to anti-bodies) both to the cells of the nervous parenchyma and the adventitia of intracerebral vessels.

The last observation seems significant. It promotes access to a much-disputed question (4): Namely, whether tissual reactions in the vascular regions in viral meningo-encephalitides are to be considered merely as reactive products of damage to the central nervous parenchyma, or whether they could represent independent primary reactions to the pathogen. Based on findings demonstrated herein, we prefer the second interpretation for experimental infection of the suckling mouse with ECHO-9 virus.

Another phenomenon among our tests results deserves underlining:
While the virus titer in the CNS tissue of the infant mouse rose to
appreciable levels (to $TCID_{50} = 10^{3.5}$) only after several passages,
showing the antigen labeling in the CNS tissue to be analogous to the
registered virus content, this was not the case in skeletal muscle
tissue. In contrast to the scant antibody fixation of the first suckling
mouse passages, the virus titer was fairly high from the start ($TCID_{50} =$
$10^{5.5-6.5}$). Intenser fluorescence appeared only with the development of
radical morphological changes. We do not consider this to be a non-
specific optical effect, since no such increase in fluorescence was noted
upon utilization of a labeled poliomyelitis antibody. In our opinion,
the interpretation of this circumstance remains in the category of a
hypothetical discussion. We therefore restrict ourselves to the recording
of the observed phenomenon: Namely, that the optically surveyable linkage
of fluorescence-labeled antibodies to ECHO-9 virus depends less upon the
level of a titrable pathogen concentration in the tissue of infected
suckling mice, and more upon a strengthened pathogenicity of the virus
for the infected host.

Discussion

F. Mueller (Duesseldorf): The impressive illustrations by
Mr. Gaedeke have shown that it is possible to visualize ECHO virus, type
9, directly in infected cells and tissues by means of fluorescein-labeled
antibodies.

We have attempted, during the past months, to demonstrate and to
localize ECHO virus, type 9, in infected tissue cultures by the method
explained to you by Mr. Klein, i.e., by the use of fluorescein-labeled
anti-complement. I can show you the feasibility of this process by way
of two photographs. The first shows a labeled polio-I virus-antibody
complex; the second, ECHO virus, type 9, as antigen-antibody complex in
the infected monkey kidney cell. You can see that the cell nucleus has
been spared, i.e., that virus propagation takes place within the cyto-
plasm.

The method using fluorescein-labeled anti-complement, as described
by Mr. Klein, has the definite advantage of requiring a single labeled
antiserum for studies of different types of virus. This fact alone would
not have justified my remark, however. I should like to propose the
following: The anti-ECHO-9 immune serum used in our fluorescence-
microscopic tests proved strictly negative against two complement-fixing
antigenic principles at dilution 1:10 in the complement fixation reaction
with Kolmer's technique. This means that this test batch showed no
deficit in the lytic power of two complement principles against the
sensitized ram erythrocytes serving as indicators. The test procedure
described demonstrates in fluorescence tests that complement must have
been labeled in amounts visible fluorescence-optically. We have found,
therefore, that this method, new to virology, can demonstrate an occult

or hemolytically inert complement fixation and conclude from the indicated comparative studies that the method is far more sensitive than the customary technique of complement fixation.

Illustrations

Fig. 1. Skeletal musculature from proximal portion of the lower extremities and the m. iliopsoas of the suckling mouse. Labeling of unfixed frozer. sections with fluorescent ECHO-9 antiserum according to Coons and Kaplan's method. -- a) 6 days after infection with ECHO-9 virus strain K 696 passed through tissue culture. Muscle fiber with isolated finely granular, green-fluorescent particles in longitudinal orientation. About 500 X; subsequently enlarged to about 1200 X. -- b) 6 days after infection with the 1st infant mouse passage of ECHO-9 virus strain K 696. Findings as in a). Muscle fiber with longitudinally striated, locally limited fluorescence. About 500 X; subsequently enlarged to about 1500 X. -- c) 6 days after infection with the 2nd suckling mouse passage of ECHO-9 virus strain K 696. String of pearls arrangement of finely granular regions of fluorescence within a muscle fiber. About 500 X; subsequently enlarged to about 1200 X. -- d) 6 days after infection with the 3rd infant mouse passage of ECHO-9 virus strain K 696. More intense, finely lumped or granular fluorescence covering larger fibrous areas in part. About 500 X. -- e) 6 days after infection with the 4th suckling mouse passage of ECHO-9 virus strain K 696. Lumpy-granular fluorescence of larger areas of muscle fiber, partly with striated arrangement within the individual muscle fibers. About 500 X. -- f) 6 days after infection with the 5th suckling mouse passage of ECHO-9 virus strain K 696. Similar findings as in e), but more pronounced. About 500 X. -- g/h) 6 days after infection with the 6th infant mouse passage of ECHO-9 virus strain K 696. Individual muscle fibers with finely lumped, longitudinally striated fluorescent regions. About 500 X; subsequently enlarged to about 800 X.

Fig. 2. Cerebral tissue of the suckling mouse. Labeling of unfixed frozen sections with fluorescent ECHO-9 antiserum by Coons and Kaplan's method. -- a) 6 days after s.c. infection with the 1st suckling mouse passage of ECHO-9 virus strain K 696. Cerebral cortex and leptomeninx above the oral striatum. No fluorescence. About 125 X. -- b) 6 days after s.c. infection with the 2nd infant mouse passage of ECHO-9 virus strain K 696. Cerebral cortex and leptomeninx above the oral striatum. Focal fluorescence in a local monocytic meningeal focus of infiltration Strong fluorescence of individual cells. About 125 X. -- c) 6 days after s.c. infection with the 3rd suckling mouse passage of ECHO-9 virus strain K 696. Cerebellum/area striata. Dense accumulation of intensely fluorescent cells in the monocytically infiltrated meninges (compressed by technical treatment). About 125 X. -- d) 6 days after s.c. infection with the 4th infant mouse passage of ECHO-9 virus strain K 696. Cerebral cortex and leptomeninx above area striata. Disseminated foci with intensely fluorescent meningeal cells with diffuse lympho-monocytic

infiltration of the lossened meninges. About 125 X. — e) 6 days after
s.c. infection with the 5th infant mouse passage of ECHO-9 virus strain
K 696. Ganglion cell group of the cerebral cortex above the oral
striatum. Multiple dense intracellular foci of fluorescence. About
500 X. — f) 6 days after s.c. infection with the 6th suckling mouse
passage of ECHO-9 virus strain K 696. Cortex in the region of the oral
striatum. Numerous intensely fluorescent cells in the central nervous
parenchyma —— partly ganglion cells (lower right), partly adventitial
cells of vessels (center). About 125 X.